Rad8Rad5/Mms2–Ubc13 ubiquitin ligase complex controls translesion synthesis in fission yeast

Article (Unspecified)

Rad8^{Rad5}/Mms2-Ubc13 Ubiquitin Ligase Complex Controls Error-freeTranslesion Synthesis in *Schizosaccharomyces pombe*

Stéphane Coulon *, Sharada Ramasubramanyan , Carole Alies*, Gaëlle Philippin*, Alan Lehmann and Robert P Fuchs [1]*

* CNRS, UPR 3081, Genome Instability and Carcinogenesis
Conventionné par l'Université d'Aix-Marseille 2, 13402 Marseille cedex 20, France
Genome Damage and Stability Centre, University of Sussex Falmer, Brighton BN1 9RQ, UK

[1] Corresponding author: fuchs@ifr88.cnrs-mrs.fr

Running head: Rad8^{Rad5} / Mms2-Ubc13 promotes TLS

Abstract

Many DNA lesions cause pausing of replication forks at lesion sites thus generating gaps in the daughter-strands that are filled-in by post replication repair (PRR) pathways. In *S. cerevisiae*, PRR involves Translesion Synthesis (TLS) mediated by Polη or Polζ, or Rad5-dependent gap-filling via a poorly characterized error-free mechanism. We have developed an assay to monitor error-free and mutagenic TLS across single DNA lesions in *S. pombe*. For both main UV- photolesions, we have delineated a major error-free pathway mediated by a distinct combination of TLS polymerases. Surprisingly, these TLS pathways require enzymes needed for poly-ubiquitination of PCNA as well as those required for mono-ubiquitination. For pathways that require several TLS polymerases the polyubiquitin chains of PCNA may facilitate their recruitment via specific interactions with their multiple ubiquitin binding motifs. These error-free TLS pathways may at least partially account for the previously described poly-ubiquitination dependent error-free branch of PRR. This work highlights major differences in the control of lesion tolerance pathways between *S. pombe* and *S. cerevisiae* despite the homologous sets of PRR genes these organisms share.

Key words : Translesion Synthesis, Ubiquitin ligase Rad6-Rad18, Ubiquitin ligase Rad8^{Rad5}/Ubc13-Mms2, Post Replicative Repair, UV photoproducts

Introduction

The DNA damage tolerance pathways, also called Post Replication Repair (PRR), deal with blocks to replication fork progression caused by DNA lesions. These pathways suppress prolonged stalling of DNA replication, allowing lesions to be bypassed and replication to continue. There are two classes of PRR pathways, Translesion Synthesis (TLS) and DNA Damage Avoidance (DA). Although the mechanisms of DA, also referred to as template switching, are largely unknown, they involve the transfer of genetic information between chromatids. This process is thought to take advantage of the replicated undamaged sister chromatid via a template-switching mechanism and is thus deemed to be accurate. In contrast, TLS pathways involve the transient recruitment of specialized DNA polymerases capable of reading through damaged bases, an intrinsically error-prone process that causes mutations *in vivo*. In eukaryotes, five specialized polymerases have been identified that perform TLS (Prakash et al., 2005). Four of them, Polη, Polι, Polκ and REV1 belong to the Y family of DNA polymerases (Ohmori et al., 2001). The fifth, Polζ, consists of the catalytic subunit REV3 and the accessory factor REV7 and belongs to the B-family (Nelson et al., 1996). In contrast to the overall mutagenic effect of TLS, Polη is the only known polymerase to act as a tumor suppressor in humans due to its capacity to accurately replicate through CPDs (cyclobutane pyrimidine dimer), the major UV-induced lesions (Masutani et al., 2000). Mutations in the Pol gene cause the variant form of Xeroderma Pigmentosum (XP-V), which has a very high risk of sunlight-induced skin cancer (Johnson et al., 1999; Lehmann et al., 1975; Masutani et al., 1999).

In *S.cerevisiae*, epistasis analysis suggests that the PRR pathway is controlled by two master genes *RAD6* and *RAD18* and further subdivided into three sub-pathways, two TLS pathways defined by Polη and Polζ and an error-free template-switching pathway. The two crucial proteins, RAD6 and RAD18 have E2-ubiquitin conjugating and E3-ubiquitin ligase activities respectively. The target in PRR of these ubiquitinating enzymes is Proliferating Cell Nuclear Antigen (PCNA), which serves as the processivity factor for the replicative and specialized DNA polymerases. In response to DNA damage in mammals and in yeast, PCNA is monoubiquitinated at lysine (K) 164 by RAD6 and RAD18 (RAD6/RAD18 complex) (Hoege et al., 2002; Kannouche et al., 2004; Watanabe et al., 2004). In *S. cerevisiae*, it has been shown that the mono-ubiquitinated form of PCNA promotes UV and MMS-induced mutagenesis (Stelter and Ulrich, 2003). In higher eukaryotes the importance of PCNA mono-ubiquitination for the TLS pathway has been shown by the fact that specialized TLS polymerases have a stronger affinity for the mono-ubiquitinated form of PCNA (Kannouche et al., 2004; Watanabe et al., 2004) by virtue of ubiquitin-binding motifs found in all four of the Y-family polymerases (Bienko et al., 2005; Guo et al., 2006; Plosky et al., 2006). *In vitro* studies using yeast enzymes have shown the ability of Polη or Rev1 to carry out TLS stimulated by ubiquitinated PCNA (Garg and Burgers, 2005; Zhuang et al., 2008). Monoubiquitinated PCNA can subsequently be polyubiquitinated via a K63 linkage by the E2 Ub conjugating enzyme UBC13/MMS2 and the E3 Ub ligase RAD5 (Parker and Ulrich, 2009).

According to current models, the poly-ubiquinated form of PCNA favors the error-free bypass of DNA lesions by means of DA strategies (Hoege et al., 2002; Moldovan et al., 2007).

In *S. pombe*, Rhp6 and Rhp18, the homologs of the *S. cerevisiae* RAD6 and RAD18, and Rad8 (the RAD5 homolog) associated with Mms2-Ubc13 are responsible for the mono- and poly-ubiquitination of PCNA on lysine 164, respectively (Frampton et al., 2006). Although absent in *S. cerevisiae,* Polκ (*dinB*) is one of the TLS polymerases in the fission yeast together with Polη (*eso1*), Polζ (*rev3* and *rev7*) and Rev1 (*rev1*). In order to gain insight into the mechanisms of TLS pathways and their control, we designed a molecular tool that allows TLS to be monitored in fission yeast. In the present study, we analyze the genetics of TLS across the two most common UV-induced lesions, namely the TT-CPD and TT(6-4) photoproducts. For both lesions, we find that the major TLS pathway is error-free. For TT-CPD, the major TLS pathway depends upon Polη and to a lesser extent Polκ, while TLS across TT(6-4) requires Polη, Polζ and Rev1. Surprisingly TLS past either lesion is largely dependent on the ubiquitin ligase complex ($Rad8^{Rad5}$/Ubc13-Mms2) that is responsible for poly-ubiquitination of PCNA at residue K164

Results:

Development of a TLS assay in S. pombe:

Principle of the assay: The plasmids used in the present work contains a defined replication origin and a single lesion located within a short sequence heterology that allows the replication pattern of the two strands to be analyzed independently (Fig1A). In *E. coli*, when a plasmid containing a single replication-blocking lesion in one strand is replicated, functional uncoupling of the replication machinery occurs at the lesion site (Pages and Fuchs, 2003). Evidence for replicative uncoupling has also been obtained in chromosomes of *S. cerevisiae* following UV-irradiation (Lopes et al., 2006). Indeed, the polymerase that replicates the lesion-containing strand is transiently stalled at the lesion site while replication of the undamaged strand proceeds unperturbed as shown both *in* vivo (Pages and Fuchs, 2003) and *in vitro* (Higuchi et al., 2003; McInerney and O'Donnell, 2004). The undamaged strand replicates with the same kinetics as either of the strands in an undamaged plasmid (Pages and Fuchs, 2003). Such plasmids are ideal tools to monitor TLS *in vivo* as they deliver the lesion in the context of a genuine replication fork. The present plasmid assay accurately measures the efficiency of replication across a single lesion compared to the efficiency of replication of the non-damaged strand that acts as an internal standard. Indeed, in the first replication cycle, the damage-containing strand will suffer from a delay that reflects the intrinsic difficulty to bypass the lesion under investigation, while the undamaged strand replicates with unmodified kinetics. In the following cycles, the daughter strand that results from the TLS event will now undergo cycles of amplification with the same kinetics as the undamaged strand. After many cycles of replication in *S. pombe*, the replicated plasmid mixture is extracted, digested with *DpnI* to eliminate non-replicated DNA and transformed into *E. coli* for determination of the relative TLS efficiency (see below).

Specificity of the vector used in S. pombe assay:

To develop such an assay, the choice of a proper replication origin that is able to stably maintain a plasmid in *S. pombe* was critical. We chose the sequence *ARSdblet,* a bi-directional autonomous replication sequence, known to maintain plasmids as monomers during many generations in *S. pombe* (Brun et al., 1995). Avoiding multimerisation improves the transmission of the plasmid through mitosis and increases the efficiency of subsequent plasmid analysis in *E. coli.* The plasmid construct (pSP) also carries the *URA4* marker for selection in fission yeast and the *ColE1* origin and the *ampR* cassette for propagation and selection in *E. coli*.

A single TT lesion, either a CPD or 6-4 photoproduct, is located at the beginning of the *lacZ'* reporter gene opposite a short sequence heterology that serves as a genetic strand marker (Fig 1A). To prevent repair of the lesion and of the sequence heterology, the TLS assay was performed in a strain deficient for nucleotide excision repair (NER), the UV dimer endonuclease-mediated second excision repair process (UVR) in *S. pombe* and mismatch repair (MMR) (named *num* parental strain).

In this strain, genes *swi10, uve1* and *mlh1* required for NER, UVR and MMR pathways were knocked-out, respectively. Replication of the lesion-containing strand by TLS yields a functional *lacZ'* gene (blue colony in *E. coli*) whatever nucleotides are inserted opposite the TT lesion, since the TT lesion is part of an in-frame valine codon (GTT) in the N-terminal region of the *lacZ* gene that can tolerate any amino-acid substitution. In contrast, replication of the non-damaged strand yields plasmids that carry a +1 frameshift giving rise to a Lac- phenotype (white colony in *E. coli*). Our assay is not able to detect frameshift TLS events but in many different systems and organisms, frameshifts induced by UV-lesions are rare events (Lawrence et al., 1993).

Damage Avoidance events (DA) are not scored in our assay, as they would generate Lac- plasmids as a result of the presence of the local sequence heterology and would thus not be monitored as TLS events. This is discussed further below. Note that we do not suggest that this plasmid system provides an accurate reflection of all the events that might occur in genomic DNA when a replication fork encounters DNA damage. Instead we use it as a system to address a specific question, namely what are the genetic requirements of TLS.

Validation of the TLS assay:

1. Kinetics of replication of control plasmid construct. First we monitored the kinetics of replication of the pSP plasmid construct in *S. pombe*. For this purpose control lesion-free pSP plasmid was transformed into *S. pombe* cells by electroporation and incubated in selective liquid medium at 30°C. At various times, total DNA was extracted from aliquots of the culture and subjected or not to *DpnI* digestion. The kinetics of appearance of plasmid DNA replicated in *S. pombe* cells was assessed by the number of colonies formed in *E. coli* by the *DpnI*-resistant plasmid sample extracted from yeast cells. As shown in Fig 2A, colonies formed by the *DpnI*-resistant plasmid sample appeared at 19 h and increased exponentially until by 46h they comprised the vast majority of the population. These data clearly show that the plasmid construct pSP undergoes efficient replication in *S. pombe* cells. Based on these results we decided to monitor TLS after a period of growth in *S. pombe* cells of at least 72h at 30°C.

2. The two strands of the plasmid are replicated independently in *S. pombe*. For a given amount of plasmid DNA, we observe the same efficiency of colony formation in *S. pombe* for unmodified control plasmid as well as for plasmids containing the single TT-CPD or TT(6-4) lesions (Fig 2B). Our hypothesis to explain the observed results is that even if the replication of one strand is blocked by the presence of a lesion, the other, undamaged, strand gets fully replicated and amplified and will thus give rise to colonies with the same efficiency as the control, lesion-free, construct (Pages and Fuchs, 2003). This observation holds in all strains tested and again strongly supports the notion that the present plasmid system does not record DA events (see discussion below).

3. Determination of the relative TLS efficiency. We next checked the ability of our assay to monitor the synthesis of each strand of double-stranded DNA during replication. For this purpose we used the lesion-free construct pSP-(CONTROL) and scored Lac+ and Lac- colonies at the 72h time

point. In principle, in the absence of a lesion, the semi-conservative nature of DNA replication should yield equivalent amounts of progeny derived from each strand. In fact, we observed a ≈ 55/45 ratio of blue/white colonies, a ratio close to the expected 50/50 value (Fig 2C). Sequencing of individual blue and white colonies yielded the *lacZ'+* and *lacZ'-* (+1 frameshift) sequences derived from each strand of the heteroduplex plasmid construct respectively. We will define the "relative TLS efficiency" (RTE) = 2 x (number of blue colonies) / (total number of colonies). The factor 2 is introduced to take into account the replication of the undamaged strand. Thus, for an undamaged control construct, the efficiency of replication of the Lac+ strand is normalized to ≈ 100%. When the pSP contruct containing a single TT-CPD or TT(6-4) lesion was replicated in *S. pombe*, the relative TLS efficiency dropped from ≈ 100% for the lesion-free control to 34% and 1.6% respectively. This observation is in good agreement with the notion that while a CPD lesion is a moderate replication block, a (6-4) photoproduct is clearly a severe impediment to replication. Interestingly, we did not observe any bias in TLS events whether the lesion was located on the leading or the lagging strand (data not shown). This observation also suggests that replication of the two strands is uncoupled and can be considered as independent events as previously observed in *E. coli* (Pages and Fuchs, 2003).

Translesion synthesis across TT-CPD (Fig. 3).

Which DNA polymerases are involved? : In the parental strain, the relative TLS efficiency (RTE) for the TT-CPD lesion is ≈ 34%, a value that reflects a distinct though moderate replication blocking capacity when compared to the replication efficiency of lesion-free plasmid (100%). Upon inactivation of Polη the TLS efficiency decreased 3-4 fold reaching a RTE value of ≈10%. A major role of Polη in TT-CPD bypass is in good agreement with results from *S. cerevisiae* and human cells (Gibbs et al., 2005; Hendel et al., 2008). More surprisingly, inactivation of Polκ also led to a 2-fold reduction in the extent of TLS. Polη and Polκ appear to control the same pathway as no significant decrease in TLS was observed in the Polη Polκ double mutant compared to the Polη single mutant strain. Sequencing of the progeny plasmids revealed that essentially all TLS events are error-free in the parental background. Even in the Polη deficient strain, >90% of the TLS events are error-free. Given the scarcity of mutagenic bypass events (<3% of TLS events in the wild type strain), we did not analyze the genetics of the mutagenic pathway for TT-CPD. Inactivation of Polζ or Rev1 had essentially no effect on TLS past the TT-CPD lesion. However, inactivation of Polζ (*rev3Δ* strain) in the Polη Polκ double mutant significantly reduced the RTE from ≈ 10% to 4%, suggesting the existence of a minor Polζ-mediated pathway that becomes apparent in the absence of the Polη / Polκ pathway. Taken together these results suggest that the major TT-CPD bypass pathway is error-free and mediated by Polη together with Polκ. We hypothesize that while *in vitro* Polη is able to perform both the insertion and extension steps Polκ frequently contributes to the extension steps *in vivo* as also found in human cells (Ziv et al., 2009).

Role of PCNA ubiquitination: For this purpose we used strains defective in genes involved in the mono- and polyubiquitination of PCNA as well as a PCNA mutant (*pcn1-K164R*) that cannot be ubiquitinated, (Frampton et al., 2006). TLS past the TT-CPD lesion is fully abolished both in the *Δrhp18* and the *pcn1-K164R* mutant strains thus demonstrating that bypass of the TT-CPD lesion absolutely depends upon ubiquitination of PCNA. The residual low level of error-free TLS events (1-2%) that is observed in *pcn1-K164R* strain and upon inactivation of the Polη, Polκ, Polζ and Rev1 polymerases most likely reflects a small contamination of the TT-CPD construct with lesion-free oligonucleotide. Strikingly, in all three strains that inactivate poly-ubiquitination of PCNA (*Δrad8, Δubc13* or *Δmms2*) while leaving mono-ubiquitination intact (Fig S1) (Frampton et al., 2006), we observe a reduction of TLS to a level similar to that observed in the Polη / Polκ deficient strains. This unexpected finding suggests that the major Polη / Polκ pathway requires the Rad8^{Rad5}/Ubc13-Mms2 complex most likely for its role in PCNA poly-ubiquitination. Although no other targets of Rad8 ^{Rad5}/Ubc13-Mms2 are presently known, we cannot formally exclude the possibility that this ubiquitin ligase complex also targets a protein other than PCNA that is essential for TLS.

In the *Δrad8* background where PCNA can be mono-ubiquitinated but not poly-ubiquitinated, the residual level of TLS (≈6%) is further reduced to background level (≈2%) when *rad30* (Polη) is inactivated, suggesting that there is a small fraction of the Polη / Polκ-mediated TLS that can function with mono-ubiquitinated PCNA. Moreover, we can deduce that the minor Polζ-dependent pathway that becomes clearly apparent in the Polη / Polκ defective background also requires the Rad8 ^{Rad5}/Ubc13-Mms2 poly-ubiquitination complex (Fig 5A).

Translesion synthesis across TT(6-4) (Fig. 4)

For TT(6-4) the relative TLS efficiency (error-free + mutagenic) in the parental strain (≈1.6%) is at least 20-fold lower than for TT-CPD illustrating its much stronger replication blocking capacity (Fig 2C). Analysis of the molecular nature of the TLS events shows that in the parental strain, about three quarters (1.2%) and one quarter (0.4%) of the TLS events are error-free and mutagenic, respectively. Most mutations were either 3'-T to C transitions as in *S. cerevisiae* (Bresson and Fuchs, 2002) or tandem TT to CC changes (Table 2). In most strains tested, these two types of mutation occur at roughly equal proportion except for *Δubc13* and *Δrev3* strains which exhibit a significantly higher proportion of TT -> CC changes compared to TT -> TC changes (Table 2). We will analyze separately the genetic requirements of error-free and mutagenic TLS pathways across TT(6-4) lesions.

Error-free TLS pathway across the TT(6-4) lesion (Fig 4A): Inactivation of either Polη or Polζ or Rev1 led to a 4-fold reduction in TLS when compared to the parental strain. No effect is seen upon inactivation of Polκ. These data are consistent with previous *in vitro* and *in vivo* studies showing that the bypass of a TT(6-4) lesion often requires the concerted action of two polymerases. *In vitro*, it has been shown that Polη is able to insert a nucleotide opposite the 3'T of the TT(6-4) photoproduct but

unable to extend the mismatched termini while Polζ is specialized for the extension step (Johnson et al., 2001). The precise role of Rev1 is still not known but given its known interactions with Polζ and Polη, it has been suggested that Rev1 acts as a cofactor that mediates the switching between the two polymerases (Friedberg et al., 2005). The residual low level of error-free TLS events (0.2-0.4%) that is observed upon inactivation of the Polη /Polζ /Rev1 pathway and also in the *pcn1-K164R* strain most likely reflects a small contamination of the TT(6-4) construct with lesion-free oligonucleotide. In strains that affect only poly- (*Δrad8, Δubc13* or *Δmms2*) or both mono- and poly-ubiquitination (*Δrhp18* or *pcn1-K164R*) of PCNA*,* we observe a reduction in TLS similar to that found in the Polη or Polζ deficient strains suggesting that the Polη /Polζ pathway requires PCNA ubiquitination and the action of the $Rad8^{Rad5}$/Ubc13-Mms2 complex. In a Polη or Polζ deficient background, inactivation of *rad8* or *rph18* did not further reduce the level of TLS. In conclusion, the error-free TLS pathway across TT(6-4) involves the combined action of Polη Polζ and Rev1. Similarly to TT-CPD bypass it also requires the $Rad8^{Rad5}$/Ubc13-Mms2 complex most likely for its role in PCNA poly-ubiquitination (Fig 5B).

Mutagenic TLS pathway across TT(6-4) (Fig 4B): The mutagenic bypass pathway accounts for about one quarter (0.4%) of all TLS events across the TT(6-4) lesion. Inactivation of each of Pol η, or Polκ or Polζ or Rev1 did not yield a significant decrease in the efficiency of mutagenic bypass of the TT(6-4) photoproduct compared to the parental strain. Moreover, in a *pol4*Δ (Pol) strain (Gonzalez-Barrera et al., 2005) no reduction in mutagenic TLS efficiency was observed (Fig 4B). However, in a triple polymerase mutant (Polη Polκ Polζ) we see a distinct decrease in the mutagenic bypass efficiency (Fig 4B). In contrast, in *S. cerevisiae*, mutagenic TLS across TT(6-4), i.e. the mis-insertion of a G across the 3'-T, is mediated by Polη followed by Polζ extension (Bresson and Fuchs, 2002).

There was no significant reduction in TLS in any of the three strains that abrogate poly-ubiquitination of PCNA (*Δrad8, Δubc13* or *Δmms2*). However, mutagenic TLS was strongly reduced when mono-ubiquitination of PCNA was also suppressed (*Δrhp18* or *pcn1-K164R)*, indicating that this pathway requires mono-ubiquitinated PCNA but not the $Rad8^{Rad5}$/Ubc13-Mms2 ubiquitin ligase complex. Similarly, in a *Δrad30* (Polη) background, while inactivation of *rad8* did not reduce TLS, inactivation of *rph18* dramatically reduced mutagenic TLS.

In conclusion, mutagenic TLS across TT(6-4) requires the mono-ubiquitinated form of PCNA and is suppressed upon simultaneous inactivation of Polη, Polζ and Polκ (Fig 5B).

Discussion:

Recently, TLS pathways across specific UV lesions have been investigated in mice and human cells using either a gap-filling assay (Shachar et al., 2009) or an SV40-based double-stranded plasmid DNA replication assay (Yoon et al., 2009). These papers identify the nature of the specific combinations of specialized DNA polymerases involved in these pathways. In the present paper, in addition to the determination of the specific DNA polymerases involved in the bypass of these lesions in *S. pombe*, we focus on the control of these TLS pathways by the PRR genes involved in the post-translational modification of PCNA by ubiquitin in *S. pombe*.

Genetic control of TLS pathways in S. pombe (Fig 5). In the present work we describe the genetic control of TLS across two common UV-induced lesions, TT-CPD and TT(6-4), in *S. pombe*. For each lesion, the major TLS pathway is error-free and requires a specific combination of TLS polymerases, namely Polη / Polκ and Polη / Polζ / Rev1 for TT-CPD and TT(6-4), respectively. As previously found in *S. cerevisiae* and in human cells, a point mutation in the replication processivity factor PCNA at position K164, that abolishes Rad6/Rad18-mediated ubiquitination, also eliminates TLS (Hoege et al., 2002; Kannouche et al., 2004; Stelter and Ulrich, 2003). Surprisingly however, in *S. pombe*, TLS also requires a functional $Rad8^{Rad5}$/Ubc13-Mms2 ubiquitin ligase complex. This complex is known to be responsible for the formation of K63-linked poly-ubiquitin chains added onto the K164-linked mono-ubiquitin in PCNA (Parker and Ulrich, 2009; Ye and Rape, 2009). While we have not formally proven that the observed defect in TLS in *Δrad8, Δubc13* or *Δmms2* strains solely reflects a deficiency in PCNA poly-ubiquitination, this is very likely since PCNA is the only known polyubiquitination target for the $Rad8^{Rad5}$, and *rad8* and *mms2* are epistatic with *pcn1-K164R* for UV survival (Frampton et al., 2006).

Damage avoidance. Any damage avoidance events occurring in our system would not be scored, as they would result in white colonies. However we do not think that they contribute significantly in our system for the following reasons: (i) DA events, such as template switching or fork regression, require the two sister chromatids to be maintained in close proximity, a structure formed upon transient replication fork stalling. As discussed above, the plasmid probes used in the present work are fully unwound by the replicative helicase, thus not forming the intermediate structures prone to undergo DA events. (ii) If DA did indeed contribute significantly in this system and poly-ubiquitination of PCNA stimulated this process, we would expect that deletion of the genes involved in this process would result in a decrease in the number of white colonies, and therefore a consequent increase in the proportion of blue colonies. In fact we observed the opposite, a decrease in the proportion of blue colonies.

A possible model for the requirement of polyubiquitination of PCNA in TLS. The present results suggest that, in *S. pombe*, polyubiquitination of PCNA is important for TLS pathways involving two specialized DNA polymerases, as exemplified here for the error-free bypass pathways of TT-CPD and

TT(6-4) (Fig 5). How may polyubiquitination of PCNA facilitate TLS ? Although we have no biochemical evidence as yet, it is tempting to speculate that the polyubiquitin chains facilitate TLS by assisting the recruitment on PCNA of the various factors involved in lesion bypass. We propose a model referred to as the "hanging tool-belt" model in which the coordinated action of two polymerases is facilitated by the concomitant interaction of their UBM or UBZ motifs with distinct ubiquitin moieties in the poly-ubi chain on PCNA. For TT-CPD bypass Polη and Polκ would thus be recruited, while Polη and Polζ would be enlisted via Rev1 for TT(6-4) bypass. While *S. pombe* Rev1 contains three UBM motifs (two in other organisms), Polη and Polκ contain one UBZ motif (though Polκ has two in other organisms). Recruitment of proteins via multiple interactions with distinct ubiquitin moeties in K63-linked chains has gained recent experimental evidence (Sato et al., 2009; Walters and Chen, 2009). The two ubiquitin interacting motifs (UIM1 and UIM2) present in protein Rap80 (Receptor associated protein 80) were shown to interact simultaneously with two neighbouring K63-linked ubiquitin moieties, thereby resulting in recruitment of the BRCA1-Rap80 to K63-linked poly ubi chains on H2AX in response to double-strand breaks. The assembly of the proteins that will form a complex competent for TLS may thus arise from the sum of various interactions involving direct interaction between the proteins themselves (PCNA-Polymerases, Polymerases-Polymerases) and/or between ubiquitin binding sites with polyubiquitin chains.

Differences between S. pombe and S. cerevisiae in PRR control. The genetic control of TLS past an AP site in *S. cerevisiae* involves the mono-ubiquitinated form of PCNA as well as the Rad5 protein in a function that does not require its helicase or ubiquitin ligase activities (Pages et al., 2008). The involvement of Rad5 in TLS has also been observed for the bypass of TT(6-4) and G-AAF adducts (Pages et al., 2008; Zhang and Lawrence, 2005). In *S. cerevisiae*, TLS was not affected in *mms2Δ* or *ubc13Δ* strains, suggesting that the role of Rad5 in TLS is distinct from its role as a partner of the E2-E3 ubiquitin complex (Pages et al., 2008). The role of Rad5 in TLS in *S. cerevisiae* is thus likely to be a 'structural' role, Rad5 mediating the switch between two specialized DNA polymerases (Pages et al., 2008). In contrast, in *S. pombe*, error-free TLS across TT-CPD or TT(6-4) is equally affected in all three mutants (*rad8Δ* or *ubc13Δ mms2Δ*) that inactivate the E2-E3 complex (Fig 3 and 4). The notion that Rad5 in *S. cerevisiae* and Rad8^{Rad5} in *S. pombe* play distinct roles in the PRR pathways is also supported by UV survival data. Whereas in *S. cerevisiae*, the UV sensitivity of *rad5*Δ mutants is much more pronounced than that of *mms2*Δ and *ubc13*Δ strains (Gangavarapu et al., 2006), the UV-sensitivities of *rad8Δ*, *mms2*Δ and *ubc13*Δ strains are similar in *S. pombe* and the three strains are epistatic (Frampton et al., 2006).

In conclusion, the present work questions the accepted dogma for the role played by poly-ubiquitination of PCNA in postreplication repair and highlights major differences between *S. cerevisiae* and *S. pombe* in the control of post-replication repair strategies. These observations warn us against

hazardous extrapolation from *S. cerevisiae* to *S. pombe* and even more so to human cells even though these organisms share sets of homologous PRR genes.

Materials and Methods

General Techniques

S. pombe methods and media have been described in Moreno *et al* (Moreno et al., 1991).

Strains

The *S. pombe* strains used in this study are listed in table 1. Each mutant strain was checked by PCR to verify that it contained the correct genomic alteration, and its phenotype was examined to ensure its consistency with expectation. The polη deficient strain was made by replacement of the Rad30 domain of Eso1 protein by the *KanMX6* cassette. The essential Ctf7 domain of Eso1 is under the control of the *nmt* promoter. This construct has been checked by southern blot and no phenotypical differences were observed between wild-type and *⊿rad30* mutant. Rhp6 proved to be unsuitable for experimental use because of its propensity to acquire suppressor mutations. To create the strain *pcn1-K164R::KanMX6*, a DNA fragment was generated by overlap PCR containing *pcn1-K164R* (Frampton et al., 2006) fused to the *KanMX6* cassette flanked with 5'UTR and 3'UTR of the pcn-1 locus. This PCR fragment has been use to replace the wild-type copy of *pcn-1* in the *S. pombe* genome. We confirmed that the strain was UV sensitive and verified that no ubiquitinated form of PCNA was detected by Western Blot (data not shown and Fig S1B).

We performed the colony-based TLS assay in strains that were deficient for the NER, UVR and MMR (*num*). In these strains, essential genes *swi10, uve1* and *mlh1* of the NER, UVR and MMR pathways were knocked-out, respectively (Bahler et al., 1998; Sato et al., 2005). We confirmed the extreme sensitivity to low doses of UV of the *num* strain (Figure S1A) and we verified that the PCNA ubiquitination profile of PRR mutants was not affected in a *num* background (Figure S1B).

The *MGZ E. coli* strain (*LacIq ⊿lacZ(M15)lacY ::Tn10 mini-tet)* is used for the TLS assay.

Plasmid construction

Single-adducted plasmids pSP-TT-CPD and pSP-TT(6-4) were constructed by ligation of an oligonucleotide containing either a single TT-CPD dimer or a TT-(6-4) photoproduct (5'- G CAA GTT AAC ACG) into a "gapped-duplex" plasmid constructed *in vitro* as described elsewhere (Becherel and Fuchs, 1999; Burnouf et al., 1989). These plasmids contain a short sequence heterology opposite the lesion site to allow independent assessment of the replication product of the two strands (Fig 1A). Covalently closed circular constructs are isolated by centrifugation on CsCl/ethidium bromide gradients. All plasmids are pUC derivatives containing a *ColE1* origin and *ampR* cassette for *E. coli* manipulation. The plasmids also contain the bidirectional *ARSDblet* as replication origin in *S. pombe cells* (Brun et al.,

1995) and the *ura4* cassette to allow selection of transformants. The *ARSDblet* and *Ura4* cassette were amplified by PCR and inserted at the unique *NdeI* site of the pCUL+ vector serie. The UV lesion is located in the N-terminal region of the *lacZ'* gene 300 nucleotides from the *ARSDblet* thus ensuring that the lesion is present in the leading strand during DNA replication.

TLS assay

S. pombe cell were grown in YES medium to OD_{600}=0.5, then prepared for electroporation as described in (Suga and Hatakeyama, 2001). 100ng of single-lesion plasmid were electroporated into 200ul freshly prepared competent cells (Fig 1B). Electroporated cells were then plated onto selective medium lacking uracil (LAH) to allow selection of cells that have received the plasmid. Plates were incubated at 30°C until colonies appeared (approximately 72h). Transformed cells were collected and the total DNA extracted as described in (Beach et al., 1982) and subjected to *Dpn*I digestion. Plasmid DNA was then transformed into *MGZ E. coli* strain and plated onto Lac indicator plates (X-Gal/IPTG/Ampicillin/Tetracyclin LB). After an incubation of 20h at 37°C, the proportion of total TLS is determined as the number of blue colonies over the total number of colonies. Each determination is the average of three independent experiments resulting from the examination of several thousands colonies for each strain. The standard error of the mean (SEM) is represented by the error-bars.

To determine the percentage of mutagenic bypass, blue colonies are isolated and minipreparation of plasmid are performed and subjected to *Hpa*I/*Xho*I double enzymatic digestion. Because of the presence of the *Hpa*I site (gttaac) at the lesion site and of the unique *Xho*I site, double enzymatic digestion generates a 1500 bp fragment if the replication of the single-lesion plasmid has occurred in a TLS error-free manner. In case of mutagenic bypass at the lesion site, the *Hpa*I site is absent and the double enzymatic digestion generates a unique fragment of 5700bp corresponding to the *Xho*I-linearized plasmid. The extent of error-free and mutagenic TLS is thus determined as a fraction of total TLS. The nature of the mutagenic TLS events is determined by sequence analysis using the following sequencing primer: 5'gcggtgtgaaataccgcacag.

Acknowledgments

We are very grateful to Joel Huberman and Takashi Toda for the gift of the *pDblet* and the *pCR2.1 (-nat / hph)* plasmids, respectively. We wish to thank Paul Russell and Luis Blanco for the *S. pombe* strains. We thank Vincent Geli, Pierre-Henri Gaillard and Agnès Tissier for useful discussions. RF laboratory is supported by the "Agence Nationale de la Recherche (ANR-06-BLAN-0258). ARL laboratory is supported by the MRC and an EC RTN. SC is supported by the "Association pour la Recherche sur le Cancer" (ARC).

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Figure legends :

Figure 1: Outline of the TLS assay in *S. pombe*:

A. Scheme of the *pSP*-TT plasmid construct. The shuttle plasmid contains the *ColE1* origin, the *ampR* resistance cassette, the bidirectional *S. pombe ARSDblet* replication origin and the *URA4* marker for propagation and selection in *E. coli* and *S. pombe*, respectively. The single TT lesion, either a cyclobutane (CPD) or 6-4 photoproduct, is located at the beginning of the *lacZ'* reporter gene opposite a short sequence heterology. Replication of the lesion-containing strand (DAMAGED strand) by TLS yields a functional *lacZ'* gene whatever nucleotides are inserted opposite the TT lesion while the NON-DAMAGED strand carries a +1 frameshift that prevents *lacZ* expression.

B. Flow chart of the experimental design. The covalently closed circular plasmid carrying the single lesion is introduced into *S. pombe* cells by transformation. Following a 72h period of replication in the yeast cells, the plasmid is extracted, the DpnI resistant plasmid replication products are transformed into *E. coli* cells and plated on ampicillin X-gal plates. The relative TLS efficiency (RTE) across a given lesion (TLS %) is defined as the proportion of blue colonies (LacZ+) divided by the total number of colonies multiplied by two (see results paragraph). The TLS events (blue colonies) are further analyzed by sequencing to determine the respective proportions of error-free and mutagenic TLS events.

Figure 2: Validation of the TLS assay

A. Kinetics of replication of a lesion free *pSP* plasmid in *S. pombe*. At various time points, total DNA was extracted from aliquots of the culture and subjected or not to *DpnI* digestion. We monitor the kinetics of appearance of plasmid DNA replicated in *S. pombe* cells by assessing the amount of colonies formed in *E. coli*. The plasmid replicated in *S. pombe* is represented by *DpnI* treated sample (black bar) and the total amount of plasmid extracted (no DpnI treatment: grey bar). At time point 46h, essentially all plasmids extracted from *S. pombe* are resistant to DpnI digestion, proving that they have been replicated in the yeast cells.

B. *S. pombe* transformation efficiency with control, TT-CPD and TT(6-4) plasmids in the parental *num*WT strain.

C. TLS assay in the parental *num*WT strain with lesion-free control plasmid (CONTROL) and plasmids carrying a single TT-CPD or TT(6-4) lesion. The relative amounts of white and blue colonies are determined in *E. coli* following replication in *S. pombe* during 72h. The proportions expressed are % is indicated on top of each bar. Error bars result from at least three independent experiments. As expected, with the lesion-free control plasmid both strands yield a similar number of plasmid progeny. We have no explanation for the slight bias (55:45) that is observed. With the lesion containing

constructs, the proportion of blue colonies dramatically decreases, reflecting the replication blocking potential of the lesions (see text).

Figure 3: Genetic control of the error-free TLS pathways across TT-CPD in *S. pombe*. The relative TLS efficiency is monitored in various strains carrying mutations in genes coding for DNA polymerases specialized in TLS or in genes affecting the ubiquitination status of PCNA or combination mutants. For sake of clarity, the strains are grouped with respect to the status of PCNA ubiquitination. Error bars result from at least three independent experiments. For TT-CPD essentially all TLS events (>99%) are error-free in the parental strain. For all tested strains, the efficiencies of transformation lie within a range comprised between 5 to 10.10^5 colonies/ g of DNA.

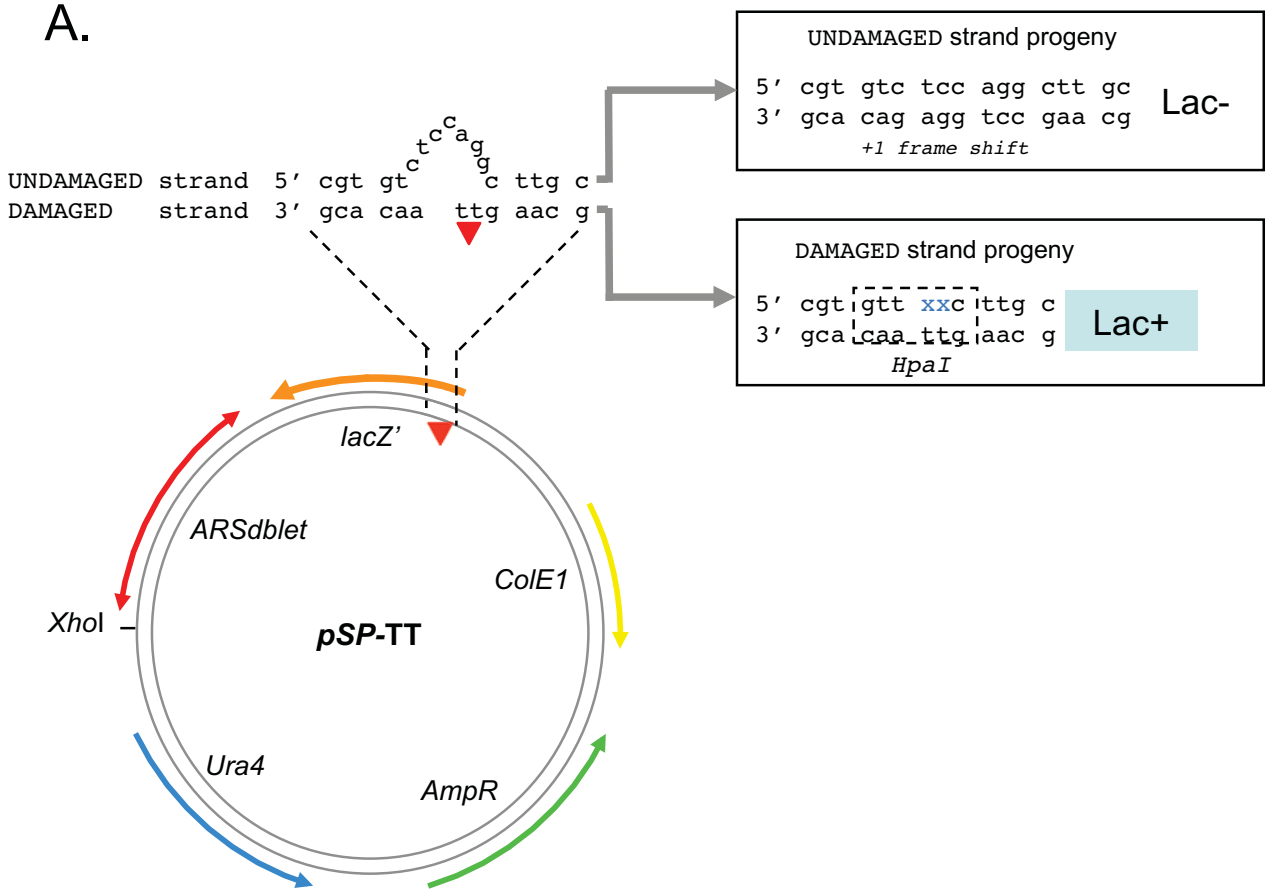
Figure 4: Genetic control of TLS pathways across TT(6-4) in *S. pombe*. the relative TLS efficiency is monitored in various strains as described in figure 3. Error bars result from at least three independent experiments. For all tested strains, the efficiencies of transformation lie within a range comprised between 5 to 10.10^5 colonies/ g of DNA.
A. Error-free TLS pathway across TT(6-4).
B. Mutagenic TLS pathway across TT(6-4).

Figure 5: The "hanging tool-belt" model: recruitment of multiple TLS polymerases by PCNA poly-ubiquitination in *S. pombe*. The major TLS pathways across TT-CPD (panel A) or TT(6-4) (panel B) lesions in *S. pombe* are error-free and involve distinct sets of TLS polymerases: Polη and Polκ for TT-CPD, Polη, Rev1 and Polζ for TT(6-4). These pathways strictly depend upon ubiquitination at residue K164 of PCNA via the Rad6/Rad18 complex. Moreover, these TLS pathways require a functional $Rad8^{Rad5}$/Ubc13-Mms2 complex that is known to attach K63-linked polyubiquitin chains to mono-ubiquitinated PCNA. We propose that in addition to the known interactions of these polymerases with PCNA (or among themselves), their interaction with the K63-linked polyubiquitin chain of PCNA facilitates their assembly into multi-polymerase complexes involved in TLS. In *S. pombe,* Polη and Polκ possess one UBZ domain, while Rev1 contains three UBM domains. Several minor TLS pathways are also inferred from careful analysis of the genetics data.

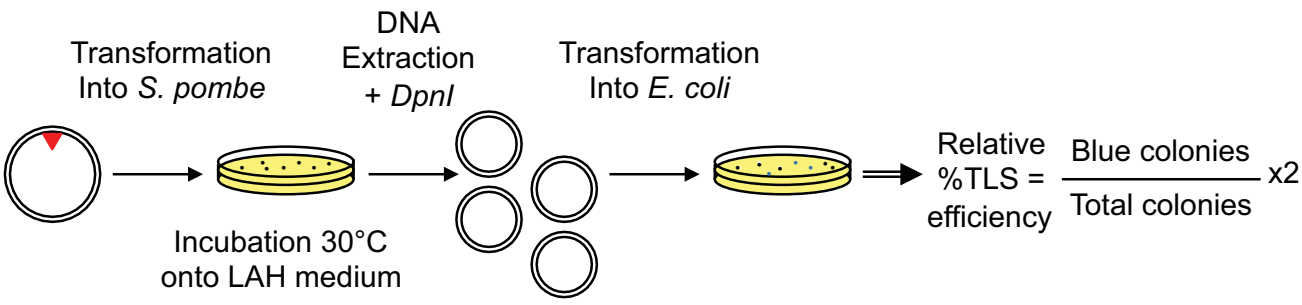
Legend Figure S1:
A. Fivefold serial dilutions of *S. pombe* strains were spotted on YES agar medium. Plates were then exposed to the indicated doses of UV light before being incubated at 30°C for 72h. The *num*WT strain in which essential genes *swi10, uve1* and *mlh1* of the NER, UVR and MMR pathways respectively were knocked-out displays an extreme sensitivity to low doses of UV.

B. The *num* genetic background does not affect the PCNA ubiquitination profile. PCNA is detected as it as been previously described (Frampton et al., 2006). The PCNA ubiquitination profile is identical in WT, *pcn1*-K164R, Δ*rhp18,* Δ*rad8*, Δ*ubc13* and Δ*mms2* strains in a wild-type or *num* genetic backgrounds in normal condition or in presence of 10mM HU. We concluded that the *num* genetic background does not modify the PCNA ubiquitination profile.

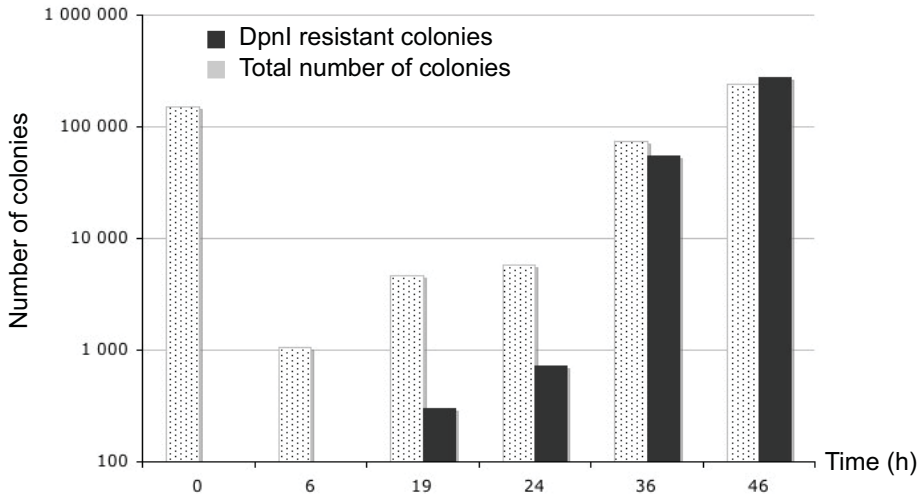
A.


UNDAMAGED strand progeny
5′ cgt gtc tcc agg ctt gc
3′ gca cag agg tcc gaa cg
+1 frame shift
Lac-
DAMAGED strand progeny
5′ cgt gtt xxc ttg c
3′ gca caa ttg aac g
HpaI
Lac+
UNDAMAGED strand 5′ cgt gt c ttg c
DAMAGED strand 3′ gca caa ttg aac g
lacZ′
ARSdblet
ColE1
XhoI
pSP-TT
Ura4
AmpR

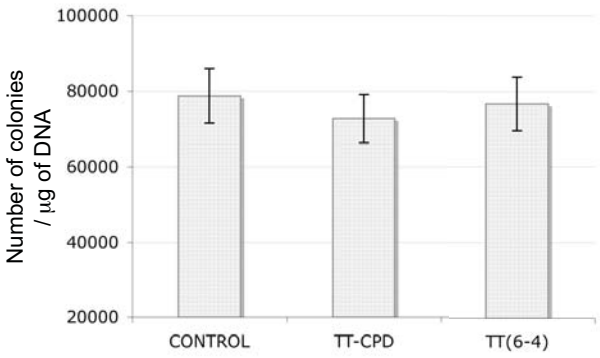
B.


Transformation
Into S. pombe
DNA
Extraction
+ DpnI
Transformation
Into E. coli
Incubation 30°C
onto LAH medium
Relative
%TLS =
efficiency
Blue colonies
Total colonies
x2

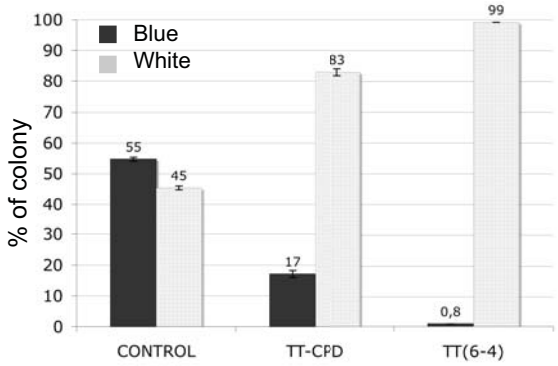
A.


1 000 000
100 000
10 000
1 000
100
Number of colonies
DpnI resistant colonies
Total number of colonies
0
6
19
24
36
46
Time (h)

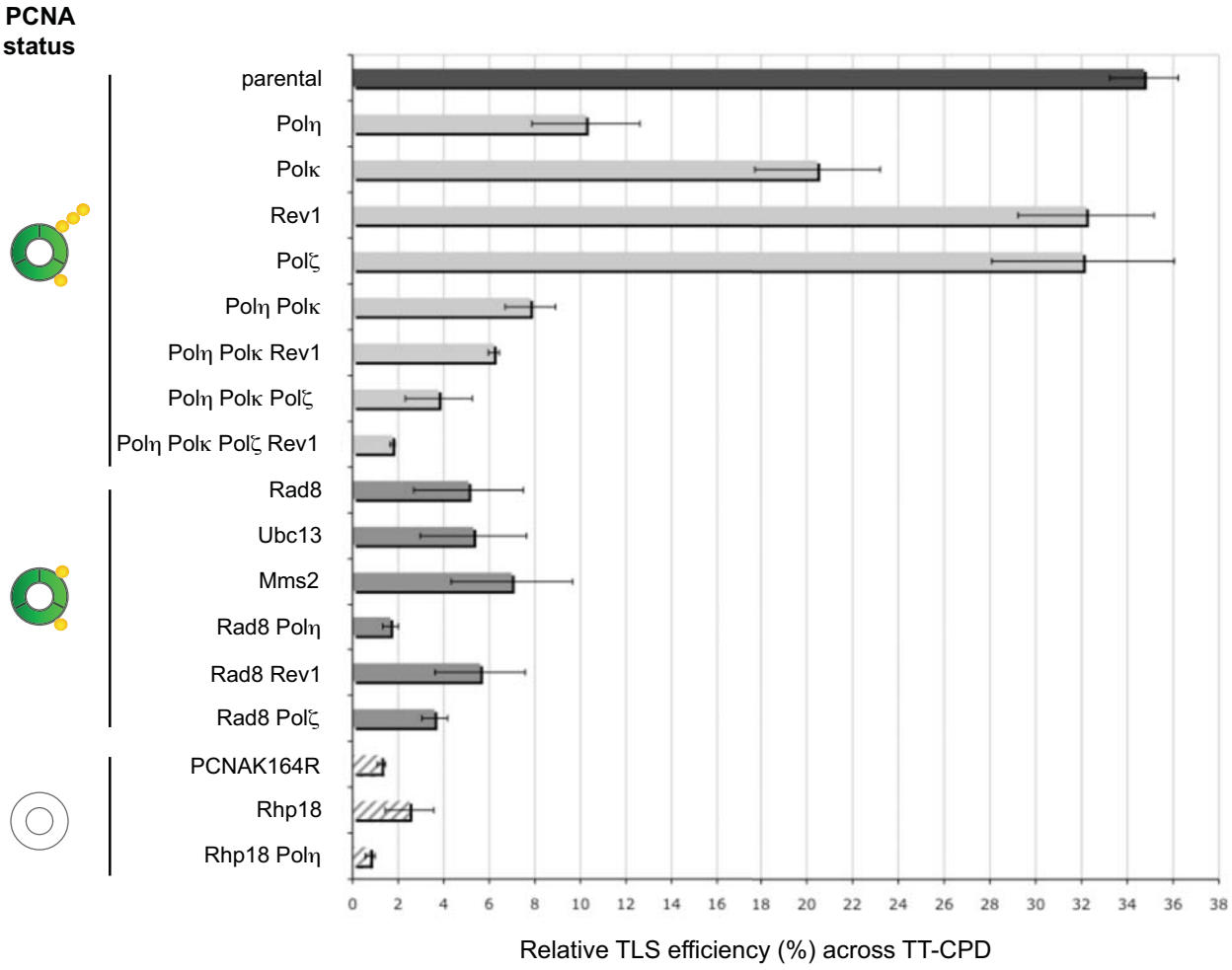
B.


100000
80000
60000
40000
20000
Number of colonies / µg of DNA
CONTROL
TT-CPD
TT(6-4)

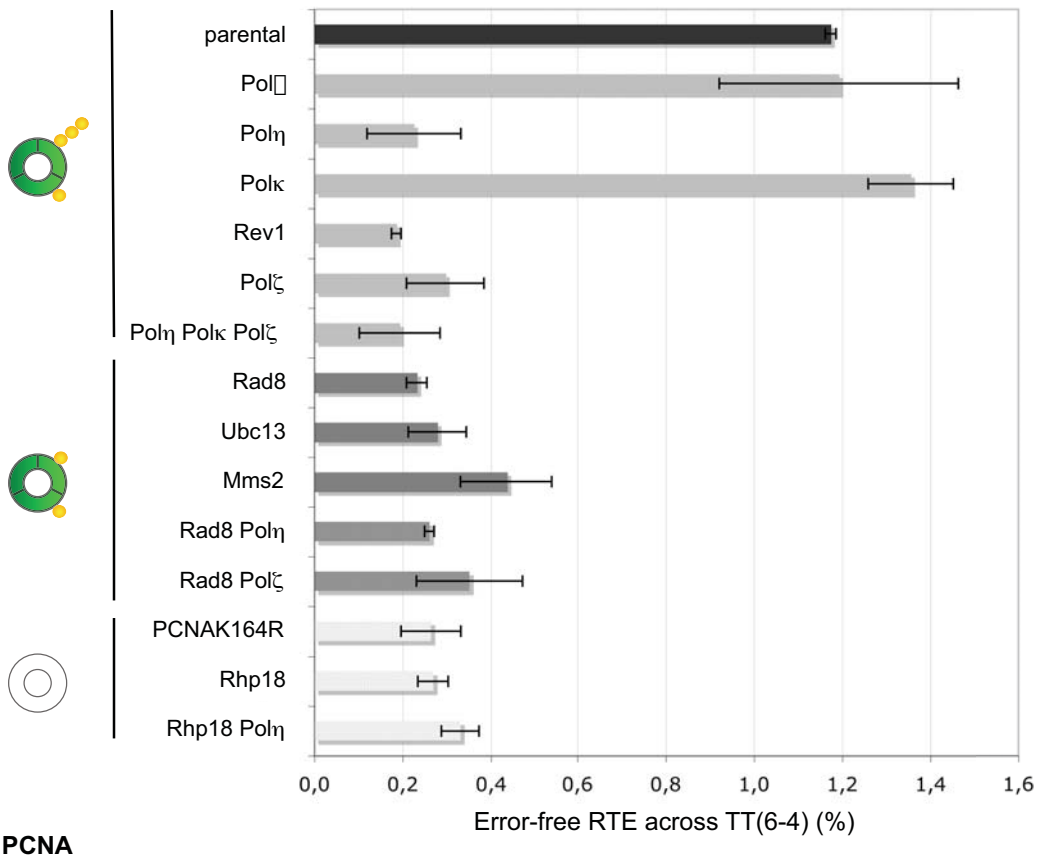
C.


100
90
80
70
60
50
40
30
20
10
0
% of colony
Blue
White
55
45
17
83
0,8
99
CONTROL
TT-CPD
TT(6-4)

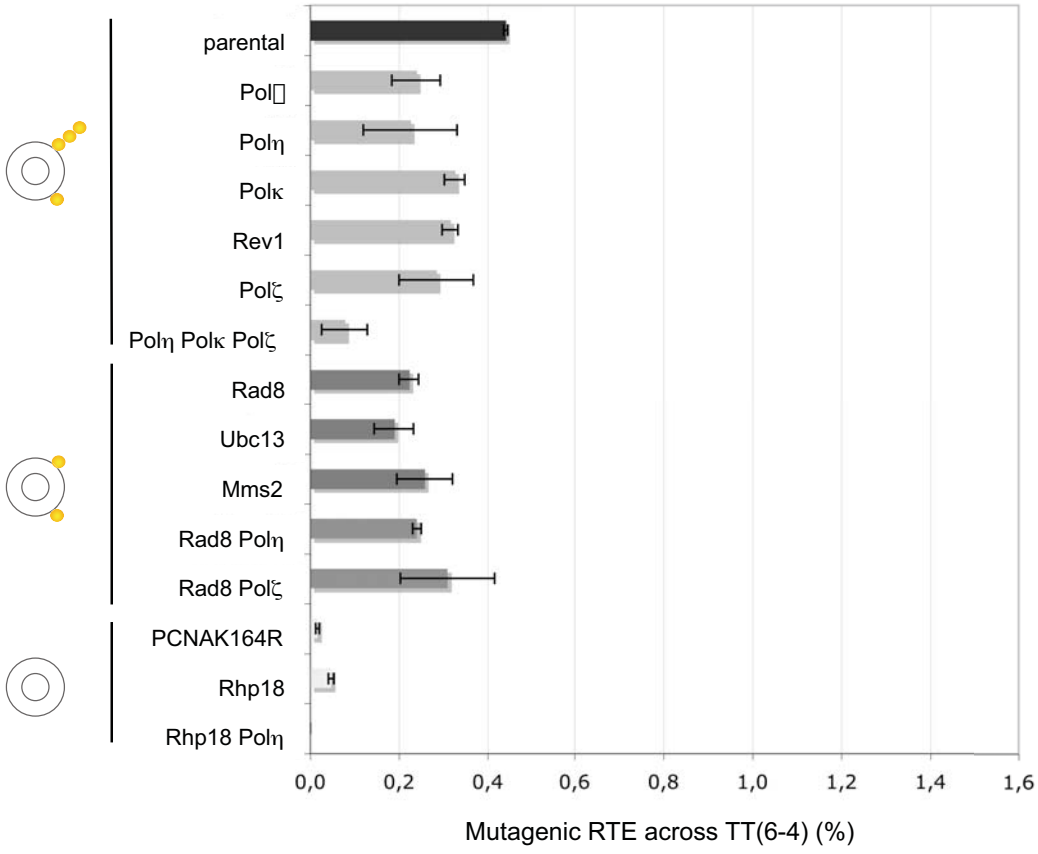
Coulon_Fig3


PCNA status
parental
Polη
Polκ
Rev1
Polζ
Polη Polκ
Polη Polκ Rev1
Polη Polκ Polζ
Polη Polκ Polζ Rev1
Rad8
Ubc13
Mms2
Rad8 Polη
Rad8 Rev1
Rad8 Polζ
PCNAK164R
Rhp18
Rhp18 Polη
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38
Relative TLS efficiency (%) across TT-CPD

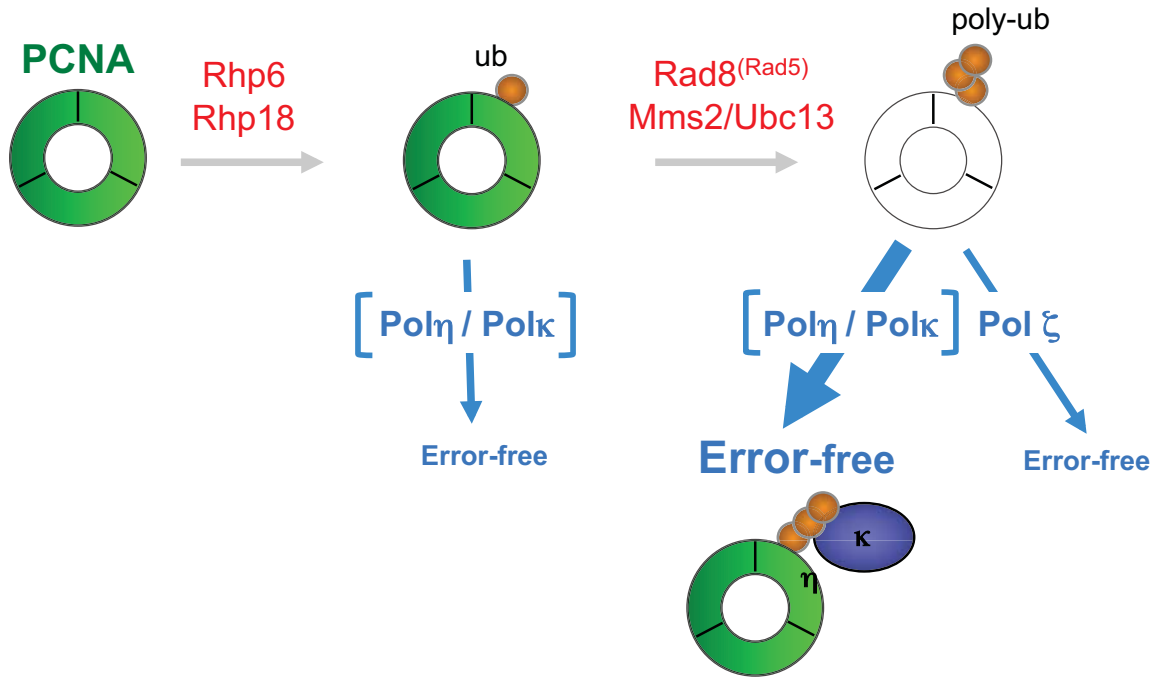
A.


PCNA status
parental
Polη
Polκ
Rev1
Polζ
Polη Polκ Polζ
Rad8
Ubc13
Mms2
Rad8 Polη
Rad8 Polζ
PCNAK164R
Rhp18
Rhp18 Polη
0,0
0,2
0,4
0,6
0,8
1,0
1,2
1,4
1,6
Error-free RTE across TT(6-4) (%)

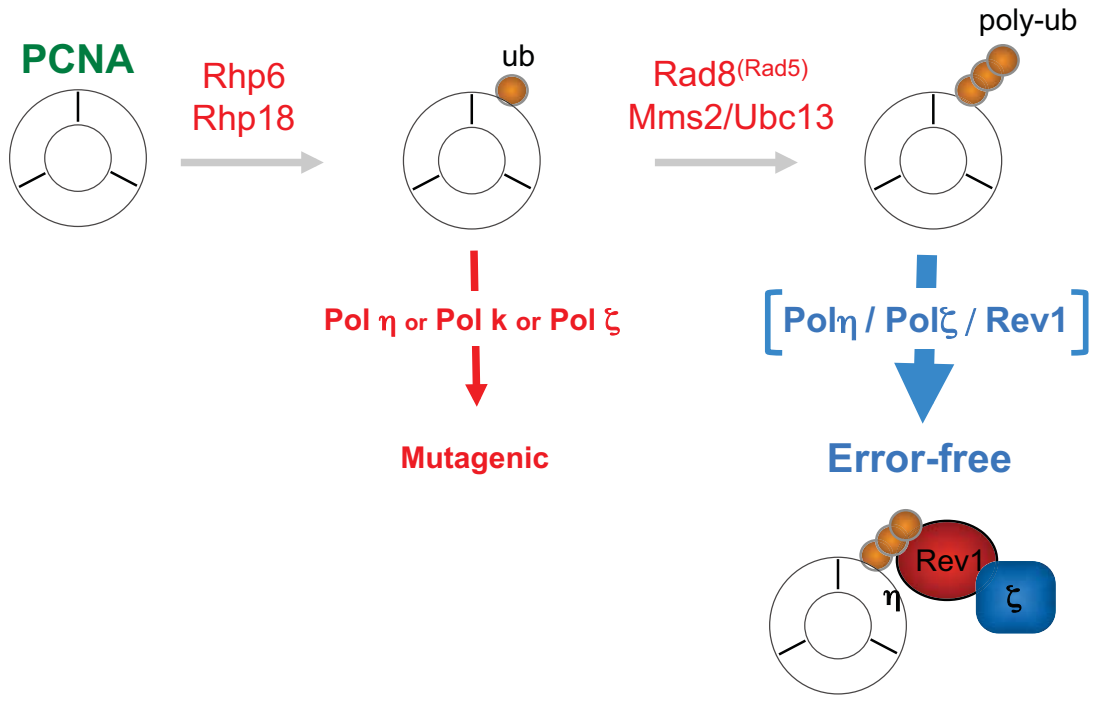
B.


PCNA status
parental
Polη
Polκ
Rev1
Polζ
Polη Polκ Polζ
Rad8
Ubc13
Mms2
Rad8 Polη
Rad8 Polζ
PCNAK164R
Rhp18
Rhp18 Polη
0,0
0,2
0,4
0,6
0,8
1,0
1,2
1,4
1,6
Mutagenic RTE across TT(6-4) (%)

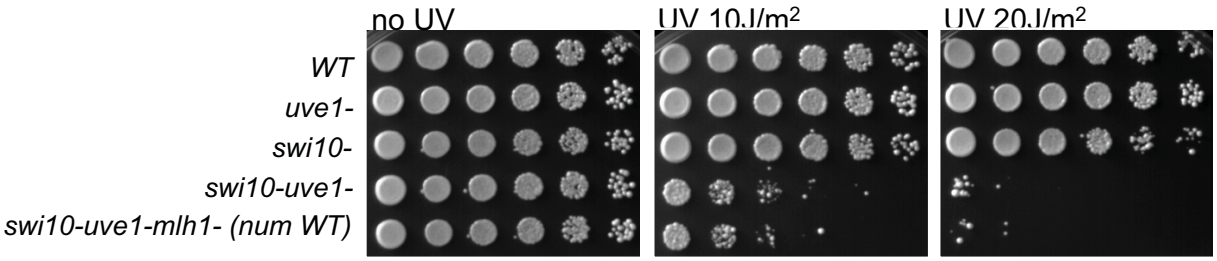
A. TLS across TT-CPD



B. TLS across TT(6-4)



A.


no UV
UV 10J/m2
UV 20J/m2
WT
uve1-
swi10-
swi10-uve1-
swi10-uve1-mlh1- (num WT)

B.

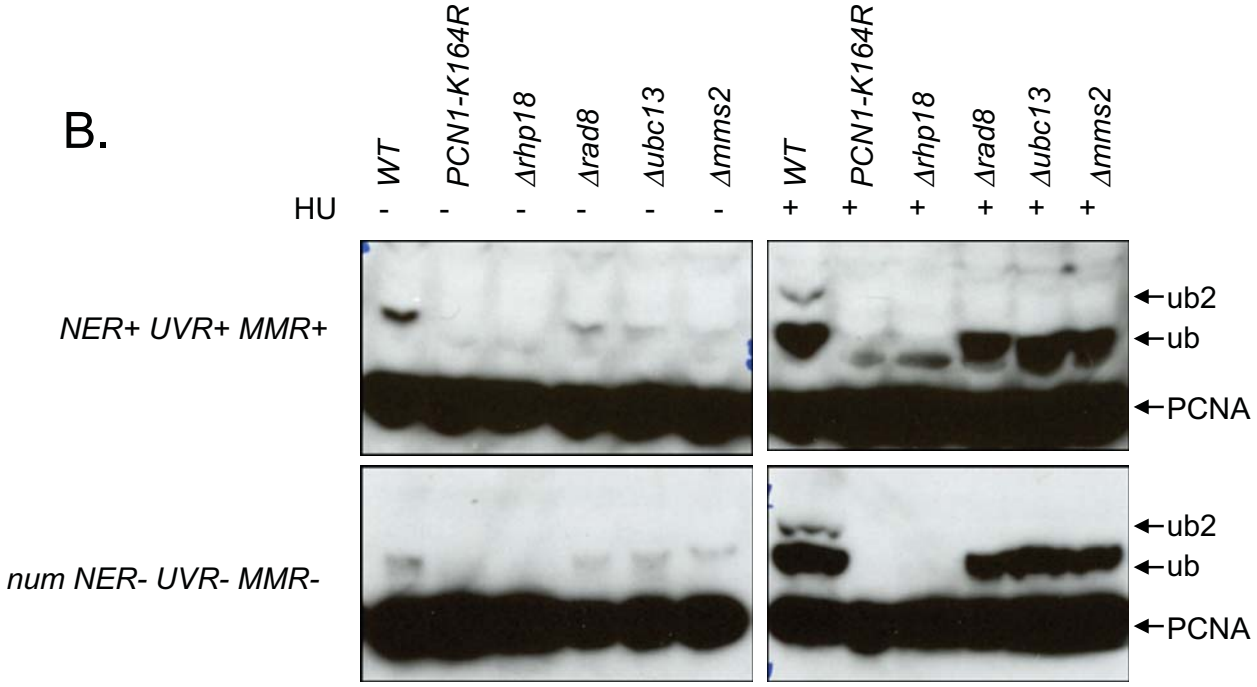

HU
- WT
- PCN1-K164R
- Δrhp18
- Δrad8
- Δubc13
- Δmms2
+ WT
+ PCN1-K164R
+ Δrhp18
+ Δrad8
+ Δubc13
+ Δmms2
NER+ UVR+ MMR+
num NER- UVR- MMR-
ub2
ub
PCNA
ub2
ub
PCNA

Table I : *S. pombe* strains used in this study

Strain	Disruption	Origin
PR109 wild type	none	*P. Russell*
SC323 *pcn1-K164R*	*pcn1-K164R::kanMX6*	this study
SC248 *Δrhp18*	*rhp18::hph*	this study
SC210 *Δrad8*	*rad8::hph*	this study
SC196 *Δubc13*	*ubc13::kanMX6*	*Frampton, 2006*
SC277 *Δmms2*	*mms2::kanMX6*	*Frampton, 2006*
SC215 *ΔdinB*	*dinB::kanMX6*	this study
SC217 *Δrev1*	*rev1::kanMX6*	this study
SC216 *Δrev3*	*rev3::kanMX6*	this study
SC260 *Δrad30*	*rad30:: kanMX6 – nmt eso1*	*Kai, 2003*
SC206 *Δuve1*	*uve1::leu2*	*P. Russell*
SC220 *Δswi10*	*swi10::hph*	this study
SC224 *Δswi10 Δuve1*	*swi10::hph uve1::leu2*	this study
SC229 *Δswi10 Δuve1 Δmlh1*	*swi10::hph uve1::leu2 mlh1::kanMX6*	this study
SC256 *Δswi10 Δuve1 Δmlh1 Δrhp18*	*swi10::hph uve1::leu2 mlh1::kanMX6 rhp18::hph*	this study
SC336 *Δswi10 Δuve1 Δmlh1 pcn1-K164R*	*swi10::hph uve1::leu2 mlh1::kanMX6 pcn1-K164R::kanMX6*	this study
SC241 *Δswi10 Δuve1 Δmlh1 Δrad8*	*swi10::hph uve1::leu2 mlh1::kanMX6 rad8::hph*	this study
SC266 *Δswi10 Δuve1 Δmlh1 Δubc13*	*swi10::hph uve1::leu2 mlh1::kanMX6 ubc13::kanMX6*	this study
SC286 *Δswi10 Δuve1 Δmlh1 Δmms2*	*swi10::hph uve1::leu2 mlh1::kanMX6 mms2::kanMX6*	this study
SC272 *Δswi10 Δuve1 Δmlh1 Δrad30*	*swi10::hph uve1::leu2 mlh1::kanMX6 rad30::kanMX6*	this study
SC251 *Δswi10 Δuve1 Δmlh1 ΔdinB*	*swi10::hph uve1::leu2 mlh1::kanMX6 dinB::kanMX6*	this study
SC252 *Δswi10 Δuve1 Δmlh1 Δrev1*	*swi10::hph uve1::leu2 mlh1::kanMX6 rev1::kanMX6*	this study
SC254 *Δswi10 Δuve1 Δmlh1 Δrev3*	*swi10::hph uve1::leu2 mlh1::kanMX6 ubc13::kanMX6*	this study
SC370 *Δswi10 Δuve1 Δmlh1Δpol4*	*swi10::hph uve1::leu2 mlh1::kanMX6 pol4::kanMX6*	this study
SC295 *Δswi10 Δuve1 Δmlh1 Δrad30 ΔdinB*	*swi10::hph uve1::leu2 mlh1::kanMX6 rad30::kanMX6 dinB::kanMX6*	this study
SC359 *Δswi10 Δuve1 Δmlh1 Δrad8 Δrad30*	*swi10::hph uve1::leu2 mlh1::kanMX6 rad8::kanMX6 rad30::kanMX6*	this study
SC362 *Δswi10 Δuve1 Δmlh1 Δrad8 Δrev1*	*swi10::hph uve1::leu2 mlh1::kanMX6 rad8::kanMX6 rev1::kanMX6*	this study
SC363 *Δswi10 Δuve1 Δmlh1 Δrad8 Δrev3*	*swi10::hph uve1::leu2 mlh1::kanMX6 rad8::kanMX6 rev3::kanMX6*	this study
SC360 *Δswi10 Δuve1 Δmlh1 Δrad30 Δrhp18*	*swi10::hph uve1::leu2 mlh1::kanMX6 rad30::kanMX6 rhp18::hph*	this study
SC378 *Δswi10 Δuve1 Δmlh1 Δrad30 ΔdinB Δrev1*	*swi10::hph uve1::leu2 mlh1::kanMX6 rad30::kanMX6 dinB::kanMX6 rev1::kanMX6*	this study
SC381 *Δswi10 Δuve1 Δmlh1 Δrad30 ΔdinB Δrev3*	*swi10::hph uve1::leu2 mlh1::kanMX6 rad30::kanMX6 dinB::kanMX6 rev3::kanMX6*	this study
SC383 *Δswi10 Δuve1 Δmlh1 Δrad30 ΔdinB Δrev1 Δrev3*	*swi10::hph uve1::leu2 mlh1::kanMX6 rad30::kanMX6 dinB::kanMX6 rev1::kanMX6 rev3::kanMX6*	this study

All strains are derivatives of the *ura-D18, leu1-32* genotype

Table II : Mutagenesis spectrum in response to TT(6-4) bypass

Strain	Number of colonies analyzed	Error-free Colonies	Mutagenic Colonies	Sequence 5'⇒3'
num parental	33	24	9	TT to TC (6) TT to CC (3)
num rad30-	20	10	10	TT to TC (5) TT to CC (3) TT to CC+ (2)
num dinB-	31	25	6	TT to TC (3) TT to CC (3)
num rev1-	27	10	17	TT to TC (11) TT to CC (6)
num rev3-	47	23	24	TT to TC (4) TT to CC (19) TT to CC+ (1)
num rad8-	49	25	24	TT to TC (12) TT to CC (12)
num ubc13-	47	28	19	TT to TC (2) TT to CC (16) TT to AC (1)
num mms2-	23	13	10	TT to TC (5) TT to CC (4) TT to AC (1)
num K164R	*17*	*16*	*1*	TT to CC (1)
num rhp18-	27	23	4	TT to TC (1) TT to CC (3)

(+) additional mutations in the vicinity of the lesion